ON LINE DATA SUPPLEMENT

SPECIFIC CONTRIBUTION OF METHIONINE AND CHOLINE IN NUTRITIONAL NONALCOHOLIC STEATOHEPATITIS: IMPACT ON MITOCHONDRIAL S-ADENOSYL-L-METHIONINE AND GSH*

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Running head: Methionine and choline in steatohepatitis.

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SUPPLEMENTAL METHODS

Measurement of ROS and GSH- ROS generation using chloromethyl-2',7'-dichlorodihydrofluorescein diacetate and GSH levels by HPLC in either cytosol or mitochondria were determined as reported previously (1).

Measurement of mitochondrial order parameter- Order parameter was determined from the fluorescence anisotropy of TMA-DPH-labelled mitochondria using polarizing filters in both excitation and emission planes as described (2, 3). Corrections for light scattering and intrinsic fluorescence was routinely made by subtracting the signal obtained from identical but unlabeled samples and the fluorescence of the buffer plus label alone, representing <5% for DPH. The order parameter was determined from steady-state fluorescence anisotropy as described previously (4).

Total, free and esterified cholesterol-The amount of cholesterol incorporated in mitochondria was measured by high performance liquid chromatography. For total cholesterol determination, 10 mg of protein were saponified with alcoholic KOH in a 60 °C heating block for 30 min. No cholesterol was detectable in the remnant protein. After the mixture had cooled, 10 ml of hexane and 3 ml of distilled water were added and shaken to ensure complete mixing. Appropriate aliquots of the hexane layer were evaporated under nitrogen and used for cholesterol measurement. High performance liquid chromatography analyses were made using a Waters µBondapak C18 10-µm reversed-phase column (30 cm x 4 mm inner diameter), the mobile phase was 2-propanol/acetonitrile (50:50, v/v) at a flow rate of 1 ml/min. Free cholesterol was assayed by directly injecting 2-propanol-extracted mitochondrial lipids on HPLC, as described (5).

PC and PE determination- The livers of rats fed MCD, MD or CD diets were perfused with saline and homogenized in chloroform:methanol:water (1:1:0.3, v/v/v). Phase separation was achieved by the addition of chloroform and 0.15 M KCl to give a final ratio of 3/2/1. The lipids were extracted and PC and PE were quantified by HPLC using a silica column Kromasil 100 Si 5mm, 20x0.46 cm (Teknokroma) as described (6).

SAM and SAH analyses by HPLC- SAM and SAH were determined from deproteinized extracts (w/v 1:1 with perchloric acid 0.5M) by HPLC, using a 5-µm Hypersil C-18 column (250 × 4.6 mm) as described (7). The isocratic elution was carried out with 40 mM ammonium phosphate, 8 mM heptane sulfonic acid, and 6% acetonitrile, pH 5.0 adjusted with 70% perchloric acid at a flow rate of 1.0 ml/min. The UV detector (Model 996, Waters) was set at 254 nm. SAM and SAH in biological samples were co-chromatographed with authentic standards and identified according to

their retention time. Standards of SAM and SAH (1mM) were dissolved in 0.5 M $HClO_4$ solution, and then diluted with 0.05 M $HClO_4$ to final concentrations.

SUPPLEMENTAL DISCUSSION

In this study, we describe a critical role of methionine in nutritional NASH. Our data is consistent with emerging findings showing the essential nature of methionine in longevity and fertility. Indeed, recent data showed that methionine supplementation in flies potentiates fertility without reducing longevity (8) and that its dietary restriction in a hypercaloric diet extends life span (9, 10). While the lack of methionine as shown in the present study leads to NASH and progressive liver disease, excessive methionine intake is highly toxic resulting in tissue injury (11) and liver damage has been observed in patients with inborn errors of metabolism associated with hypermethioninemia (12). Moreover, recent findings have shown that methionine supplementation resulted in stimulated mitochondrial ROS generation and free radical leaking in liver but not in heart mitochondria (13), changes associated with increased SAM and SAH content which underlie the selective hepatotoxicity of hypermethioninemia. Thus, based on the previous and present data, it appears that a critical level of methionine intake is needed to ensure proper hepatic function and mitochondrial membrane integrity and antioxidant defense

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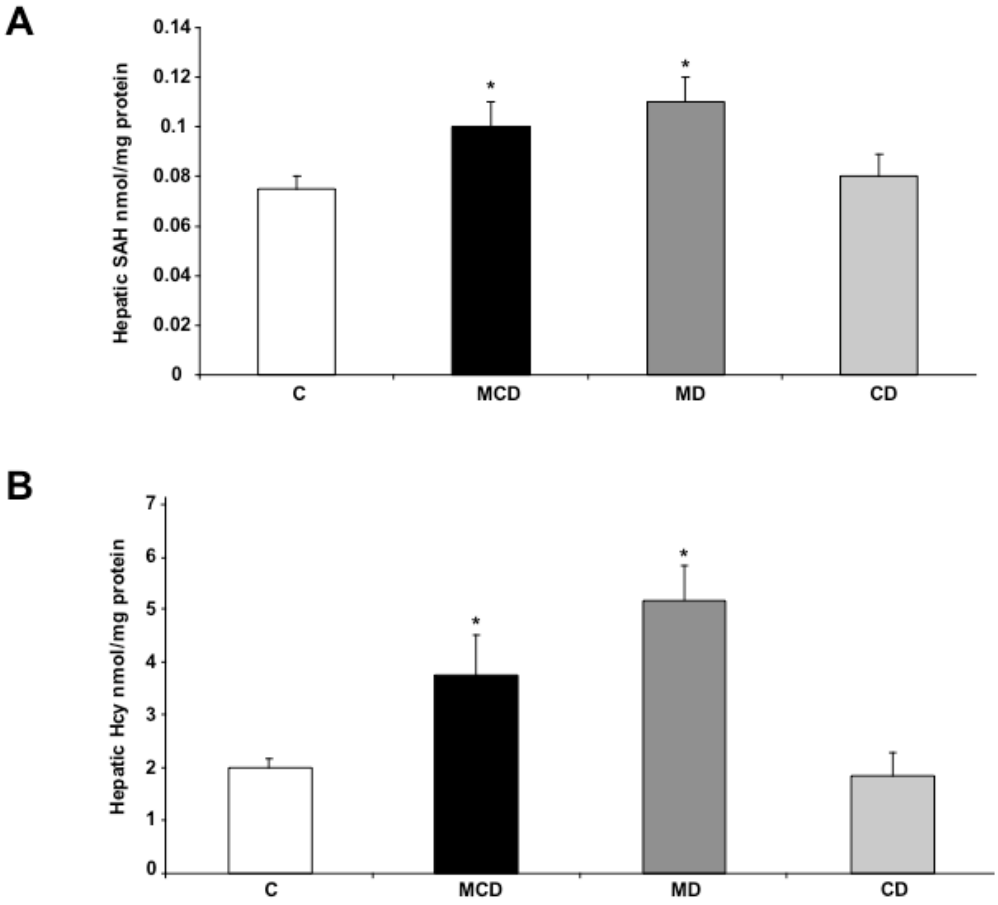
SUPPLEMENTAL FIGURES

Supplemental Figure 1. Hepatic SAH and homocysteine levels in mice fed MCD, MD or CD. Mice fed the corresponding dietary regimes were sacrificed after 15 days. Liver extracts were obtained and processed for SAH and homocysteine determinations by HPLC as described in the on-line data supplement. Data are the mean of mean±SD of 4-6 individual mice. *$p<0.05$ vs control mice C.

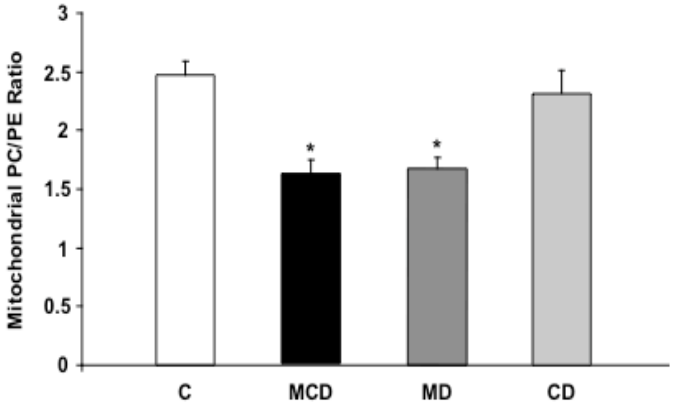
Supplemental Figure 2. Mitochondrial PC/PE ratio. Mitochondrial fractions isolated from the different groups of mice fed MCD, MD or CD, were treated with chloroform:methanol to isolate total lipid extracts. PC and PE were resolved and quantitated by HPLC as described in the on-line data supplement methods. Data are the mean±SD of 4-6 individual mice. *$p<0.05$ vs control mice C.

Supplemental Figure 3. Protection against NASH by SAM therapy. Mice were fed MCD or MD diets for 15 days and in some cases mice were treated with SAM at day 7 for an additional week. Livers were processed for mitochondrial GSH (A), mitochondrial SAM (B), serum ALT (C) or histology (D). Data are the mean±SD of 4-6 individual mice. *$p<0.05$ vs control mice C. ** $p<0.05$ vs the corresponding group without SAM.

Supplemental Figure 1. Caballero et al.



Supplemental Figure 2. Caballero et al.



Supplemental Figure 3. Caballero et al.